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14. ABSTRACT
TSC is a common inherited predisposition syndrome, affecting nearly 1 in 7,500 individuals. Individuals with TSC develop benign tumors in multiple organs, including the retina, skin, lung, kidney and brain. The identification of valid targets in TSC has been discouraging. In search of TSC targets, we recently identified NPM as a downstream effector of mTOR signaling in TSC cells, providing cells with an abundant supply of ribosomes necessary for supporting their increased growth rate. We now provide evidence that NPM forms a novel complex with DDX5 to drive TSC cell growth. Using the NCI diversity set and Maybridge chemical compound sets, we have now identified two compounds that potently inhibit split-luciferase activity in two TSC cells lines. Notably, these two compounds also inhibit the proliferation of TSC/p53-null and UMB1949 TSC cells while not altering the growth rates of p53-null cells that maintain TSC function, suggesting that these compounds might specifically target NPM-DDX5 complex formation when it is enhanced in TSC cells. We have also shown that these two compounds inhibit the formation of endogenous NPM-DDX5 complexes in TSC cells.

15. SUBJECT TERMS
NPM, DDX5, TSC, chemical library, split-luciferase

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1. INTRODUCTION

TSC is a common inherited predisposition syndrome, affecting nearly 1 in 7,500 individuals. Individuals with TSC develop benign tumors in multiple organs, including the retina, skin, lung, kidney and brain. However, these lesions can present as malignant. TSC results from mutations in either the TSC1 or TSC2 gene, resulting in hyperactivation of the mTOR-signaling pathway. This pathway ultimately controls downstream effectors of cellular mRNA translation and proliferation. Multiple studies in mice have demonstrated the requirement of mTOR activity for aberrant TSC cell growth in vitro and in vivo and these initial findings have translated to human cells as well. However, the identification of other valid targets in TSC has been discouraging, leaving the field to concentrate on rapamycin and its analogues as the sole treatment for TSC. In search of additional TSC targets, we recently identified NPM as a downstream effector of mTOR signaling in TSC cells, providing cells with an abundant supply of ribosomes necessary for supporting their increased growth rate. We now provide evidence that NPM forms a novel complex with DDX5 to drive TSC cell growth. Targeting this interaction might provide a novel treatment strategy for TSC patients.

2. KEYWORDS

NPM, DDX5, TSC, chemical library, split-luciferase

3. ACCOMPLISHMENTS

Major Goals of the Project

There were three major goals for this grant proposal: 1) Screen 2,000 chemical library for split luciferase activity, 2) Screen 14,400 chemical library for split luciferase activity, and 3) validate that inhibitors block NPM-DDX5 complex.

Goals Accomplished

In the first year of this grant application, we have now successfully set up the high throughput split luciferase screening model and scaled it up to 96-well plate format using a non-automated screening method. Using *TSC1/p53*-null mouse embryonic fibroblasts (MEFs) and UMB1949 cells, we have screened the NCI Diversity Set of compounds for chemicals that inhibited luminescence by 3-fold or greater. We successfully used *p53*-null cells as a negative control on any resultant hits. Concentrations for each compound were 5 mM. Cells were incubated for 24 hours in each compound prior to incubation with luciferin. All cells were stably transduced with a GFP expression construct to account for cell viability as well as split-luciferase activity in each well of cells. Each plate contained a negative control where the ratio of GFP to photon flux was set at 100%. This allowed us to calibrate all 96 wells on each individual plate without the risk of plate-to-plate variance. An example of the results obtained for each plate is shown in Figure 1 that depicts plate 11. Samples #29 (75%), #77 (82%), and #92 (88%) resulted in significant decreases in luminescence.

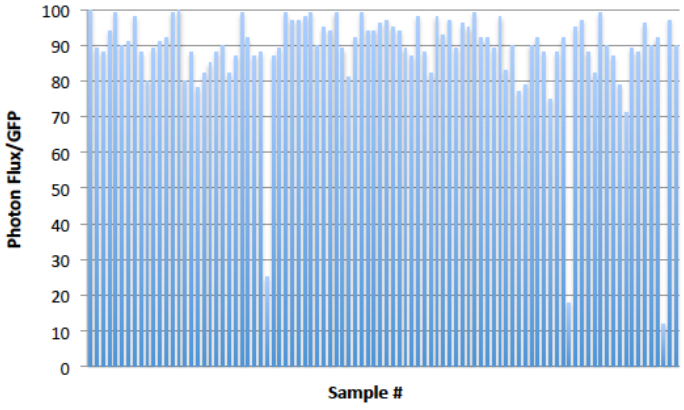

Figure 1. Analysis of Plate #11. 96-well plate #11 was seeded with TSC1/p53-null MEFs that had been transduced with GFP expression constructs and the NPM-DDX5 split-luciferase constructs. Cells were incubated for 24 hours in 5mM of each compound and analyzed for GFP fluorescence and luciferase luminescence. Lane 1 corresponds to DMSO control and set at 100%.

We next sought to determine whether any of these three compounds (tested at 5μM concentration) could selectively inhibit the growth of TSC cell lines while not altering the proliferation of other non-TSC cells. Compound #29 and compound #77 were quite potent inhibitors of *TSC1/p53*-null MEFs and UMB1949 cells while leaving *p53*-null cells proliferation largely intact (Figure 2) over a three-day period of growth. However, compound #92 inhibited the proliferation of both TSC cells and *p53*-null cells where TSC1 is competent (Figure 2), suggesting that its effects are largely toxic and not specific for any TSC-dependent biology.

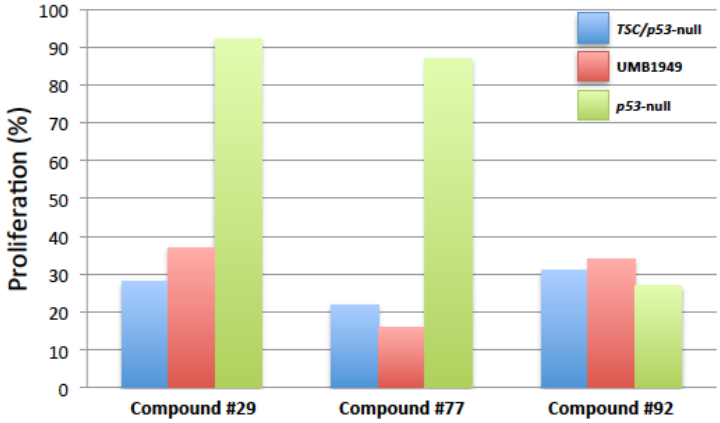

Figure 2. Three test compounds result in inhibition of cell proliferation. We plated three cell lines: *TSC1/p53*-null, UMB1949, and *p53*-null cells in 6-well dishes and incubated each well with DMSO control or 5μM of each compound (#29, #77 and #92). Cells were counted three days later and normalized to DMSO control wells.

Our first set of experiments for the second and final year of this grant were to initiate and compete the proposed 14,400 Maybridge chemical libraries screen. We were able to perform this screen rapidly since we had spent much time working out the necessary conditions for a robust luciferase signal in our split NPM-DDX5 complex split luciferase construct. Unfortunately, the larger library screen did not produce any bona fide NPM-DDX5 complex inhibitors. We initially had 42 compounds that resulted in significantly decreased luciferase activity in our split luciferase readout assay. However, 30 of these compounds did not reproducibly inhibit luciferase when tested a second time. The remaining 12 compounds did not inhibit the formation of NPM-DDX5 interactions as observed in co-immunoprecipitation experiments. Thus, we returned our focus back to compounds #29 and #77.

We conducted a more thorough response for cell proliferation with each compound. In Figure 3, we show that both compounds are capable of inhibiting the long-term proliferation of TSC/p53-null cells as measured by foci formation.

Figure 3. Two test compounds inhibit proliferation. *TSC1/p53*-null cells were plated in a 100mm dish and incubated with 5μM of compound #29 or #72 or DMSO control. Cells were treated every three days with fresh media containing compounds. Resulting cell colonies were fixed and stained with Giemsa to visualize colonies.

Our final goal was to determine whether compound #29 and #72 inhibit the formation of NPM-DDX5 complexes by using immunoprecipitation assays with NPM-recognizing antibodies. We cultured *TSC1/p53*-null cells and treated with DMSO, compound #29, or compound #72 at 5μM for 24 hours. Treated cells were harvested, lysed and incubated for 1 hour with antibodies recognizing NPM. The immune complexes were precipitated with Protein A Sepharose beads and proteins separated by SDS-PAGE. The resultant immune complexes were immunoblotted

with antibodies recognizing NPM and DDX5. Input control shows levels of each protein are consistent throughout the experiment. Cells treated with DMSO exhibit formation of NPM-DDX5 complexes (Figure 4). However, treatment with compound #29 or #72 resulted in a dramatic reduction in the amount of DDX5 protein bound to NPM (Figure 4). These results suggest that compounds #29 and #72 disrupt the formation of NPM-DDX5 complexes in TSC1/53-null cells.

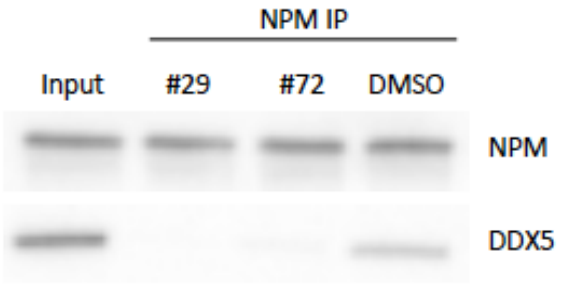

Figure 4. Monitoring NPM-DDX5 complexes in cells. *TSC1/p53*-null cells were plated in a 100mm dish and incubated with 5μM of compound #29 or #72 or DMSO control for 24 hours. Cells were harvested, lysed and immunoprecipitated with NPM antibodies. Immunoblot analysis of the immune complexes was performed using antibodies recognizing NPM and DDX5.

Training Opportunities
Nothing to Report

Results Disseminated to the Community
I participated this past year in disseminating our initial findings to three independent groups of large donors to the American Cancer Society. These donors visited my laboratory at Washington University where I discussed the research in this grant proposal and how our results were moving the field of TSC research and treatments forward. We engaged in a question and answer session where the donors queried me on the clinical impact of this work. I anticipate doing this laboratory tour again next year and have already been asked by the American Cancer Society to do so.

Plans for Next Reporting Period
Nothing to Report

4. IMPACT
Impact on Principal Discipline
Our current work will be incredibly impactful for those researchers involved in identifying novel compounds that might provide evidence as to a novel approach to treat TSC clinically. Additional, our data now provide evidence that the split luciferase approach can work when looking for compounds that block the interaction of two proteins. Additionally, we have proven the utility of studying the NPM-DDX5 interaction in the context of TSC. We have successfully shown that our two major lead compounds are capable of inhibiting the proliferation of TSC-deficient cells, leading to future studies as to why targeting NPM-DDX5 complexes is so important in TSC.

Impact on Other Disciplines
Nothing to Report

Impact on Technology Transfer
Nothing to Report

Impact on Society

We have disseminated the data and ideals from this grant proposal to several groups in the St. Louis community. They were encouraged by our progress and excited about the future clinical impact our work might provide.

5. CHANGES/PROBLEMS

Changes in Approach

Nothing to Report

Anticipated Problems or Delays

We were delayed in our ability to test the 14,400 Maybridge chemical libraries for split luciferase activity. This was both a product of our inability to rapidly obtain the library and our difficulties in acquiring a robust luciferase signal that was readable above the observed noise in the system. We were able to successfully complete all of the aims for this project in the two-year timeline.

Changes in Human, Animal Biohazards and/or Selective Agents

Nothing to Report

6. PRODUCTS

Publications, Conference Papers and Presentations

Nothing to Report

Internet Sites

Nothing to Report

Technologies or Techniques

We now have two lead compounds that effectively inhibit NPM-DDX5 complex formation and also proliferation of TSC-deficient cells.

Inventions, Patents and/or Licenses

Nothing to Report

7. PARTICIPANTS

Individuals That Have Worked on Project

Name:	Jason D. Weber
Project Role:	PI
Nearest person month worked:	1.2
Contribution to Project:	Dr. Weber served as the mentor for Ms. Yaddanapudi in planning all experiments and overseeing the final data analysis.
Funding Support:	NIH R01CA190986, NIHR01CA174743, W81XWH-15-1-0528

Name:	Sree Yaddanapudi
Project Role:	Graduate Student
Nearest person month worked:	6
Contribution to Project:	Ms. Yaddanapudi performed all of the experiments outlined in specific aim1 for year

	1

Changes in Active Other Support for PD/PI
Nothing to Report

Other Organizations Involved as Partners
Nothing to Report